Biochem. J. (1971) **125**, 869–878
Printed in Great Britain

Deiodination of L-Thyroxine and its Activity on the Oxidation *in vitro* of Reduced Nicotinamide–Adenine Dinucleotide by Peroxidase plus Hydrogen Peroxide

By TRINIDAD JOLIN and GABRIELLA MORREALE DE ESCOBAR
Thyroid Research Unit, Instituto G. Marañón, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velázquez 144, *Madrid* 6, *Spain*

(*Received* 11 *March* 1971)

When L-thyroxine activates the oxidation of NADH by peroxidase+H_2O_2, little removal of phenolic-ring iodine atoms becomes apparent until most of the NADH has been oxidized, after which it increases markedly. This extensive deiodination is accompanied by loss of the ability of thyroxine to catalyse the oxidation of NADH by peroxidase+H_2O_2. The slight deiodination observed before the appearance of extensive deiodination is somewhat higher when the effect of thyroxine on NADH oxidation is greater, and lower when thyroxine has exerted a slighter effect. ICN (but not I_2 or thyronine) catalyses NADH oxidation, in both the presence and the absence of peroxidase+H_2O_2: thyroxine+peroxidase+H_2O_2 are thus comparable with ICN alone in their effects on NADH oxidation. The obvious conclusion from the above observation, namely that the active moiety is the halogen liberated from thyroxine (or ICN) is, however, not directly supported by some of the results obtained by measuring the degree of deiodination of thyroxine in the system. In an attempt to reconcile some apparently contradictory conclusions, it is suggested that, when thyroxine activates oxidation of NADH by peroxidase+H_2O_2, the diphenyl ether structure is undergoing cyclic deiodination and iodination. This would be accompanied by the maintenance in the reaction medium of an oxidized form of iodine, similar to that liberated by ICN, which would be the actual active moiety, until the NADH concentration becomes so low that the diphenyl ether structure is ruptured oxidatively. An alternative explanation is that thyroxine is oxidized to a form that either oxidizes NADH or loses iodine in competing reactions.

From studies done in this and other laboratories (Galton & Ingbar, 1962; Braverman & Ingbar, 1962; Escobar del Rey & Morreale de Escobar, 1964; Morreale de Escobar & Escobar del Rey, 1967) it would appear that the amount of thyroxine that is deiodinated *in vivo* is a good index of the strength of hormone action. From studies *in vivo*, however, no conclusion could be reached as to whether or not there is a causal relationship between deiodination and activity of thyroxine and, if so, whether deiodination is a prerequisite or a consequence of hormonal activity. It was decided to study this point in a model system in which thyroxine exerts a measurable effect *in vitro*, though obviously the results would not necessarily be directly pertinent to what happens *in vivo*.

Effects elicited by thyroid hormones *in vitro* on systems that may be related to respiratory metabolism have aroused considerable interest. Klebanoff (1959*a*,*b*, 1960, 1961, 1962*a*,*b*) has shown that L-thyroxine and some of its structural analogues catalyse the oxidation of reduced nicotinamide–adenine dinucleotides and other hydrogen donors [such as D(−)-adrenaline, DL-noradrenaline, ascorbic acid, ferrocytochrome *c*, ergothioneine] by peroxidases (horseradish, myelo- and lacto-peroxidase) in the presence of H_2O_2, or of a system that generates it. From these studies Klebanoff (1960) concluded that thyroxine was acting as an electron carrier, being alternately oxidized by the peroxidase+H_2O_2 system and reduced by the hydrogen donor. This could imply a cyclic oxidation and reduction of thyroxine or of a product of its degradation formed during the reaction. Because Klebanoff (1959*a*,*b*) did not determine what changes the thyroxine was undergoing during the reaction, the possible role of the iodine that might be liberated from the hormone could not be assessed: although the presence of iodine in the thyronine analogues seemed to confer special characteristics to the

reaction, thyronine itself appeared to be active in the system.

We report here the results obtained when studying whether the thyroxine that activates the oxidation of NADH by peroxidase (EC 1.11.1.7)+H_2O_2 is deiodinated or not during the reaction, and if there is any relationship between the deiodination of thyroxine and its activity in the system. We have also investigated whether the oxidation of NADH by peroxidase+H_2O_2 is activated by compounds containing iodine in an electronic configuration that might be similar to the one it presumably has when it is being incorporated into, or liberated from, the thyroxine molecule: Rall, Roche, Michel, Michel & Varrone (1963) have shown that the effects *in vitro* of thyroxine on swelling of liver mitochondria may be mimicked by ICN and I_2.

EXPERIMENTAL

The oxidation of NADH was measured by the decrease in E_{340} and that of D(−)-adrenaline by the increase in E_{300}. Measurements were taken in a Beckman D-U2 spectrophotometer with silica cells with a 1 cm light-path, with buffer as a blank.

Unless otherwise stated, 0.3 μmol of NADH [or 0.5 μmol of D(−)-adrenaline], 100 μg of peroxidase and 1 μmol of H_2O_2 were added to 3 ml of 0.1 M-sodium phosphate buffer, pH 7.3. The extinction was measured and 2–3 min later 0.05 μmol of thyroxine were added in a 50 μl volume. L-[^{131}I]Thyroxine was also added immediately, in a 20–60 μl volume, this volume depending on the specific radioactivity of the preparation at the time of use.

Portions (about 40 μl) were withdrawn from the reaction mixture at different intervals to assess the degree of deiodination of [^{131}I]thyroxine. These portions were immediately mixed with 0.2 ml of 1 mM-6-propyl-2-thiouracil or of whole horse serum. This was done to interrupt the deiodination reaction and to prevent non-specific deiodinations during the ensuing paper-chromatographic procedure, by the addition of a strong reducing agent (6-propyl-2-thiouracil) or of a mixture of proteins that protect the hormone by binding. To assess the degree of deiodination of [^{131}I]thyroxine and the reliability of the results obtained the following techniques were used: paper chromatography in butan-1-ol–ethanol–1 M-NH_3 (5:1:2, by vol.); precipitation with trichloroacetic acid after addition of plasma proteins; and paper electrophoresis. These techniques were described in detail by Jolin, Morreale de Escobar & Escobar del Rey (1966). The major ^{131}I-labelled spots found after paper chromatography of the present reaction mixture are [^{131}I]thyroxine and two products of the deiodination reaction, namely $^{131}I^-$ and a labelled moiety with R_F approx. 0, referred to as 'origin material', and known to be mainly iodinated proteins. The degree of deiodination of [^{131}I]thyroxine is thus assessed by calculating the proportion of the total ^{131}I recovered on the chromatogram that is still found as [^{131}I]thyroxine. The difference between this value and the one corresponding to the amount in the initial solution of [^{131}I]thyroxine gives the degree of deiodination of [^{131}I]-thyroxine by the system under study. For reasons indicated in the Results section, it is important to check the reliability of the results obtained by paper chromatography. This may be done with the other two techniques, namely precipitation with trichloroacetic acid and paper electrophoresis. With these techniques only one of the labelled reaction products, $^{131}I^-$, is separated from the remaining [^{131}I]thyroxine. The other reaction product, 'origin material', is found together with the remaining [^{131}I]thyroxine in the protein precipitate or at the point of application of the sample. Therefore, these two techniques are not suitable to measure the degree of deiodination of [^{131}I]thyroxine. But if the sum of the proportions of ^{131}I found as [^{131}I]thyroxine plus that found as 'origin material' after paper chromatography is appreciably lower than the proportions of ^{131}I found in the protein precipitated by trichloroacetic acid, or at the point of application of the sample after paper electrophoresis, it is likely that the results obtained by paper chromatography are affected by non-specific deiodinations occurring during this analytical procedure. Moreover, a systematic and large difference between the total radioactivity recovered on the paper chromatogram and that recovered on the electrophoretic strip or that added to the plasma sample before precipitation with trichloroacetic acid would also help to disclose the presence of non-specific deiodinations accompanied by loss of ^{131}I.

L-Thyroxine labelled with ^{131}I in the phenolic ring was obtained as a 50% (w/v) solution in propylene glycol from Abbott Laboratories, Chicago, Ill., U.S.A. L-Thyroxine (sodium salt pentahydrate) was obtained from British Drug Houses Ltd., Poole, Dorset, U.K., or Sigma Chemical Co., St Louis, Mo., U.S.A.; D(−)-adrenaline, 6-propyl-2-thiouracil and ICN, from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; horseradish peroxidase (type I), NADH (disodium salt, grade II), catalase (33 000 units/mg) and I_2 from Sigma Chemical Co.; and H_2O_2 from Merck Reagenzien A.-G., Darmstadt, Germany.

RESULTS

Assessment of the deiodination *in vitro* of thyroxine is difficult for technical reasons: some methods (precipitation with trichloroacetic acid and paper electrophoresis) are not suitable because one of the labelled reaction products ('origin material') is not separated from the remaining [^{131}I]thyroxine. With paper chromatography, which does separate adequately both reaction products ($^{131}I^-$ and 'origin material') from the remaining [^{131}I]thyroxine, non-specific deiodinations may occur during the analytical procedure. These might be caused by light, the presence of oxidizing agents etc. (Taurog, 1963*a*,*b*; Morreale de Escobar, Llorente, Jolin & Escobar del Ray, 1963; Jolin *et al.* 1966) and the results may be totally unrelated to the degree of deiodination actually occurring in the system under study. During preliminary experiments, the samples withdrawn from the reaction mixtures (NADH, peroxidase+H_2O_2, and thyroxine+[^{131}I]-thyroxine) were applied directly to the paper and the deiodination values obtained after chromato-

graphy were found to be much higher than those assessed by paper electrophoresis and trichloroacetic acid precipitation, though the proportion of ^{131}I found as 'origin material' had been added to that of the remaining [^{131}I]thyroxine before the results obtained by paper chromatography were compared with those of the other two techniques. In view of findings with other deiodinating systems

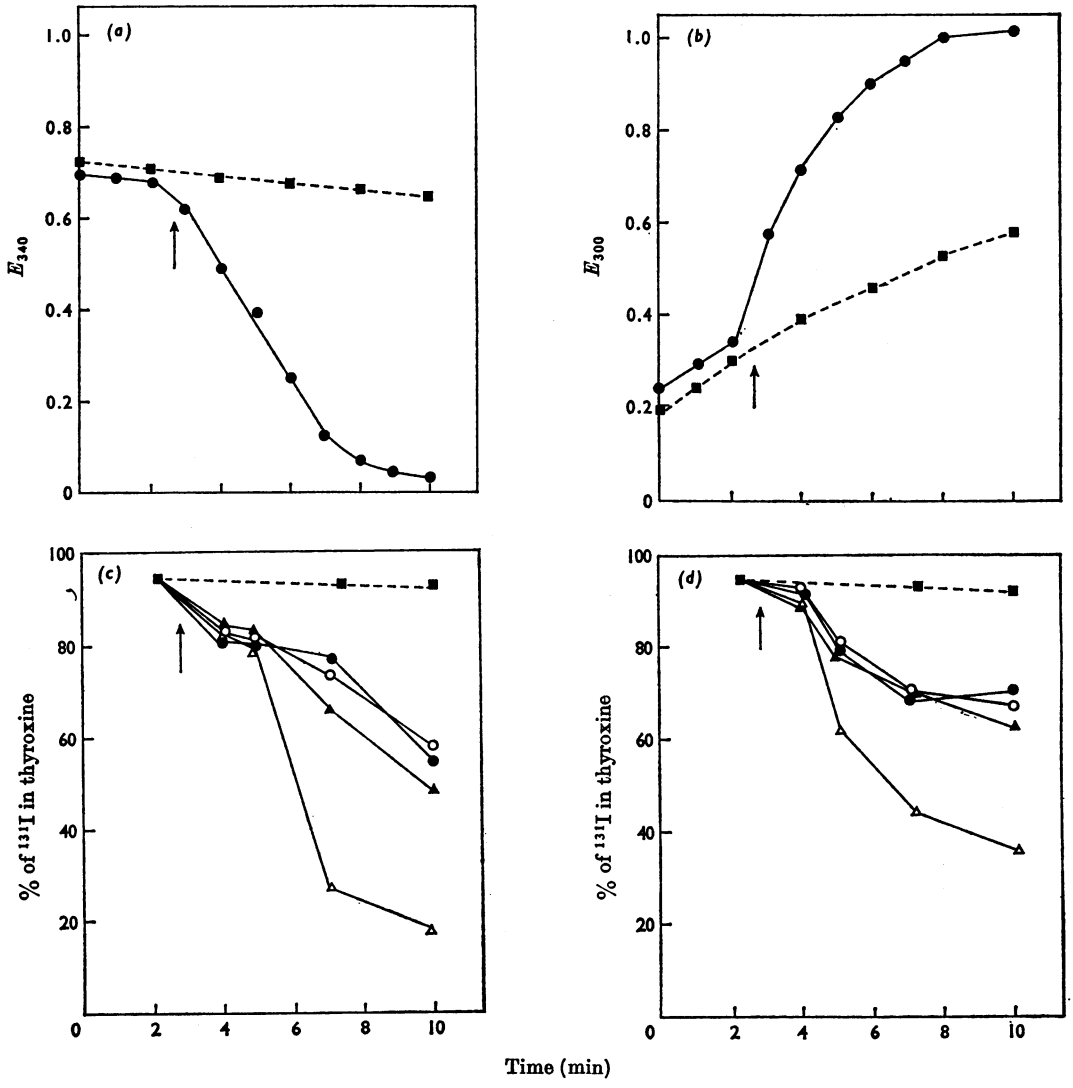

Fig. 1. Oxidation of 0.32 μmol of NADH (*a* and *c*) or of 0.5 μmol of D(−)-adrenaline (*b* and *d*) in the presence of [^{131}I]thyroxine + 0.05 μmol of thyroxine (——) or of [^{131}I]thyroxine alone (– – –), added at the times indicated by the arrows. The initial reaction mixtures contained 100 μg of peroxidase and 1 μmol of H_2O_2 in 3 ml of 0.1 M-sodium phosphate buffer, pH 7.3. (*c*) and (*d*) show the degree of deiodination of [^{131}I]thyroxine at different reaction times, as evaluated by several techniques and after the protective precautions described in the text had been taken. ●, Percentage of ^{131}I found with the proteins after precipitation with trichloroacetic acid or (○) at the point of application of the sample after paper electrophoresis. This corresponds to the remaining [^{131}I]thyroxine + one of the labelled reaction products, 'original material'. △ and ■, Percentage of ^{131}I found as [^{131}I]thyroxine after paper chromatography. ▲, Percentage of ^{131}I found as [^{131}I]thyroxine + 'original material', as assessed by the same technique. Agreement between results represented by ●, ○ and ▲ indicates that non-specific deiodinations of [^{131}I]thyroxine during paper chromatography were not affecting results, and that the results (△) were valid.

in vitro (Taurog, 1963*a*,*b*; Morreale de Escobar *et al.* 1963; Jolin *et al.* 1966), the possibility was considered that non-specific deiodinations were occurring during the chromatographic process. Thus, to avoid possible artifacts, the samples withdrawn from the reaction vessels were mixed immediately with plasma or a strong reducing agent before portions were placed on the chromatographic or electrophoretic paper strips or added to a plasma sample for precipitation with trichloroacetic acid. In this manner satisfactory agreement was obtained with the three techniques. Results illustrated in Figs. 1(*c*) and 1(*d*) show good agreement between the values of remaining [^{131}I]thyroxine+'origin material', as assessed by paper chromatography, and the proportion of ^{131}I precipitated with the proteins or at the point of application of the sample on the electrophoretic strips. These precautions were therefore used throughout the present study. All deiodination results shown or discussed are those obtained by paper chromatography, their reliability being checked by the other two techniques in the manner indicated above.

It was found that in the absence of both peroxidase and H_2O_2, thyroxine did not affect the concentration of NADH and was not deiodinated. In the absence of peroxidase, but in the presence of 0.01, 1 or 10μmol of H_2O_2, thyroxine did not induce NADH oxidation; in the absence of H_2O_2, but in the presence of 100, 200 or 300μg of peroxidase, thyroxine resulted in a slight oxidation of NADH. In none of these cases was deiodination of [^{131}I]thyroxine evident.

Fig. 1 shows the effects of thyroxine on oxidation of NADH and D(−)-adrenaline by the peroxidase+H_2O_2 system, and the degree of deiodination of [^{131}I]thyroxine at different reaction intervals, as measured by paper chromatography. It also shows how the reliability of these results is checked by trichloroacetic acid precipitation and paper electrophoresis. As the oxidation of the electron donor proceeds, the degree of deiodination of [^{131}I]thyroxine increases.

The rate of NADH oxidation by the peroxidase+H_2O_2 system in the presence of thyroxine, and the ensuing deiodination of the hormone, were studied concomitantly by using reaction mixtures with different oxidizing capacities or in the presence of inhibitors of different types. In the presence of a constant amount of NADH, the oxidizing activity of the system increases with increasing concentrations of peroxidase and, within certain limits, of H_2O_2. When the concentration of the latter is increased from 1 to 10μmol in the presence of

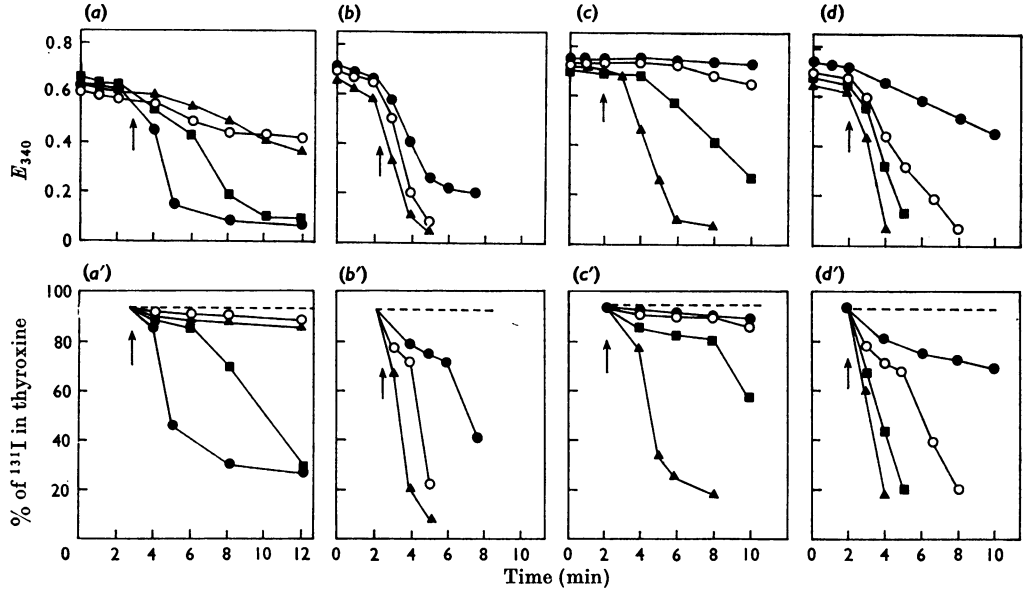

Fig. 2. Effect of addition of thyroxine on NADH oxidation under various conditions. The initial reaction mixtures contained 100μg of peroxidase and 1μmol of H_2O_2 in 3ml of phosphate buffer unless otherwise stated. (*a*) Variations of amount of H_2O_2: ○, 0.01μmol; ●, 1μmol; ■, 10μmol; ▲, 100μmol; (*b*) variation of the amount of peroxidase: ●, 100μg; ○, 200μg; ▲, 300μg; (*c*) addition of human plasma to give concentrations of 0.3% (▲), 1% (■), 2% (○) and 3% (●) of the final volume; (*d*) addition of KCN: ▲, 0; ■, 3μmol; ○, 10μmol; ●, 50μmol. (*a'*), (*b'*), (*c'*) and (*d'*) show the corresponding deiodination of [^{131}I]thyroxine, as assessed by paper chromatography; --- indicates the deiodination of the original solution of [^{131}I]thyroxine.

100 μg of peroxidase, the oxidizing activity is known to decrease, because of inhibition of the enzyme (Saunders, Holmes-Siedle & Stark, 1964). This inhibition is almost complete with 100 μmol of H_2O_2. If these inhibiting concentrations of H_2O_2 are used, the activating effect of thyroxine on NADH oxidation also decreases. Results obtained by varying the proportions of peroxidase+H_2O_2 are illustrated in Figs. 2(*a*), 2(*a'*), 2(*b*) and 2(*b'*); those obtained in the presence of human plasma or potassium cyanide at different concentrations are shown in Figs. 2(*c*), 2(*c'*), 2(*d*) and 2(*d'*). Thus, an increase in the oxidizing capacity of the system resulted in an increasing effect of thyroxine on NADH oxidation and an increasing degree of deiodination; the opposite effect was observed in the presence of inhibitors of the reaction. Though results with catalase are not shown, the presence of 16 μmol of this enzyme in the standard reaction mixture abolished the effect of thyroxine on NADH oxidation, and its deiodination. Most experiments indicated that there was more deiodination when the effect of thyroxine on NADH oxidation had been more marked. However, many of the results suggested that a possible relationship between the degree of deiodination of [^{131}I]thyroxine and the intensity of the effect of thyroxine on NADH oxidation would not be linear. Moreover, the degree of deiodination of [^{131}I]thyroxine appeared to be related to the amount of NADH remaining in the reaction mixture at the moment the sample was withdrawn and the reaction stopped, whatever the experimental conditions employed. These factors are illustrated in Fig. 3, which shows results corresponding to the different reactions shown in Figs. 2(*a*) and 2(*a'*): deiodination was not very marked until a major part of the NADH had been oxidized, and the degree of deiodination found at different reaction times appeared to be related to the E_{340} of the sample when it was withdrawn for chromatography, whatever the concentration of H_2O_2 used. Fig. 4 shows the pattern that emerged when we considered as a whole the results obtained at different reaction times by using the different experimental conditions indicated in Figs. 1 and 2 and different batches of [^{131}I]thyroxine. The degree of deiodination of [^{131}I]thyroxine again appears to be dependent on the E_{340} of the reaction mixture at the moment the sample was withdrawn for paper chromatography and that it rarely exceeded a value of 20–25% until most of the NADH had disappeared, that is, at E_{340} about 0.25–0.20 or lower. It then increased sharply, becoming quite extensive. The more marked the effect of thyroxine on NADH oxidation had been, the sooner these low E_{340} values were reached and the sooner extensive deiodination was observed. These observations suggested that the rate of deiodination of [^{131}I]-thyroxine would change sharply when most of the

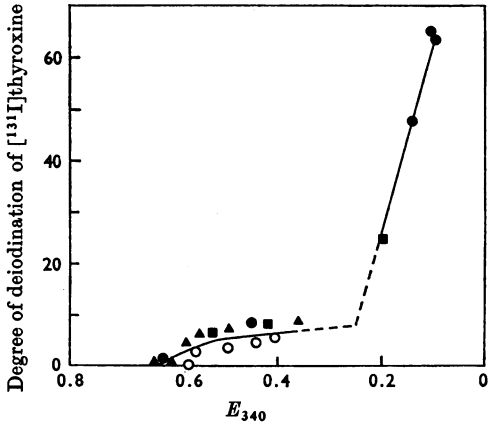

Fig. 3. Degree of deiodination of [^{131}I]thyroxine at different reaction times, corresponding to the experiment illustrated in Figs. 2(*a*) and 2(*a'*), is plotted against the corresponding E_{340}. The degree of [^{131}I]thyroxine deiodination is expressed as the difference between the proportion of ^{131}I present as [^{131}I]thyroxine in the reaction mixture and in the original [^{131}I]thyroxine solution. Irrespective of the oxidizing capacity of the system, the degree of deiodination of [^{131}I]thyroxine appears to be related to the amount of remaining NADH. The symbols are as designated for Fig. 2(*a*).

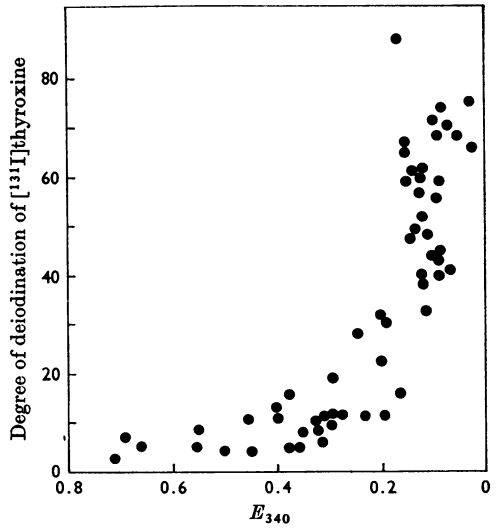

Fig. 4. Relationship between the degree of deiodination of [^{131}I]thyroxine (expressed as for Fig. 3) and the E_{340} of the reaction mixture at the moment the sample is withdrawn for paper chromatography. The results were obtained at many different reaction times under the experimental conditions described in Figs. 1 and 2.

NADH is oxidized, a pattern visible in many of the deiodination curves illustrated in Fig. 2 for which the NADH oxidation rate had not been too rapid. When activation of NADH oxidation is very strong, it is difficult to determine the actual E_{340} of the mixture at the moment when the reaction is stopped. Fig. 5 shows the change in the rate of deiodination of [^{131}I]thyroxine during a single experiment during which samples were taken at 1 min intervals.

When the oxidation of NADH by peroxidase+H_2O_2 is allowed to proceed in the presence of thyroxine until different E_{340} values are reached and a second portion of NADH is then added, the rate of oxidation of the latter depends on the E_{340} value attained before addition of the new portion (Fig. 6*a*). The rate of oxidation of the second NADH sample was smaller the further the previous oxidation of NADH had been allowed to proceed. When this had reached an E_{340} value of 0.2 or lower, the rate of oxidation of the second NADH sample was as slow as that in the absence of thyroxine. Fig. 6(*b*) shows results obtained when thyroxine is first incubated with peroxidase+H_2O_2, and NADH is then added at different time-intervals. The rate of NADH oxidation decreases as the length of the period of preincubation of the thyroxine is increased. The degree of deiodination of [^{131}I]thyroxine immediately before the addition of the second NADH sample (experiment illustrated in Fig. 6*a*), or of the first one (experiment illustrated in Fig. 6*b*), was determined and plotted against the ensuing rate of NADH oxidation. The result obtained is shown in Fig. 6(*c*): a very good correlation exists between the proportion of undergraded thyroxine remaining when the NADH is added and the ensuing rate of NADH oxidation.

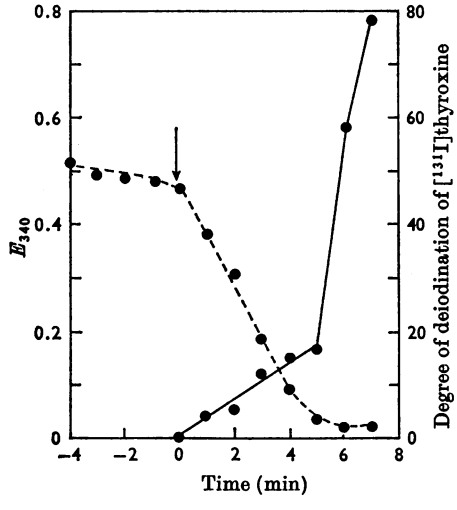

Fig. 5. Rate of oxidation of NADH by peroxidase+H_2O_2 in the presence of thyroxine+[^{131}I]thyroxine (---), added at the point indicated by the arrow, is shown together with the degree of [^{131}I]thyroxine deiodination (——) found at different reaction times and expressed as for Fig. 3. Experimental conditions were similar to those described for Fig. 1.

Thyronine in amounts equimolar with those of thyroxine, or approximately equimolar with those of NADH (Figs. 7*a* and 7*a'*), increases the rate of NADH oxidation in the presence, but not in the absence, of the peroxidase+H_2O_2 system. In this respect, the effect of thyronine appears similar to that of thyroxine. But it is clearly different from the effect of thyroxine in another very important aspect: the rate of oxidation of a second NADH sample is as low as if thyronine had not been initially added to the system, which is contrary to the result when thyroxine is used. This lack of catalytic effect of thyronine was confirmed by using several different concentrations of thyronine, and adding the second NADH sample after the reaction had been permitted to proceed to several different E_{340} values.

Addition of I_2 results in a sharp and transient increase in the rate of NADH oxidation, in both the presence and the absence of the peroxidase+H_2O_2 system (Figs. 7*b* and 7*b'*). As with thyronine, the rate of oxidation of a second NADH sample is not higher than that obtained in the absence of I_2. The I_2 does not appear to inactivate the peroxidase+H_2O_2 system: if 0.05 μmol of thyroxine is added after the transient effect of I_2 has been measured, the usual activation of NADH oxidation is observed. As already indicated, thyroxine would not have been effective in the absence of an active peroxidase+H_2O_2 system.

When ICN is added to the system, the rate of NADH oxidation increases, in both the presence and the absence of the peroxidase+H_2O_2 system (Figs. 7*c* and 7*c'*), though the effect is less marked in the latter case. The rate of oxidation observed when a second NADH sample was added was similar to the one corresponding to the oxidation of the first NADH sample. This observation suggested that the effect of ICN on the system might be catalytic, as already indicated for the effect of thyroxine. However, it might be so even in the absence of the peroxidase+H_2O_2 system, that is, in conditions where no effect of thyroxine is apparent.

A catalytic effect of ICN on NADH oxidation, in both the presence and the absence of the peroxidase+H_2O_2 system, was repeatedly observed. Results from one series of experiments are summarized in Table 1 and compared with those obtained by using thyroxine+peroxidase+H_2O_2. As may be seen, both 2 μmol of ICN and 0.05 μmol

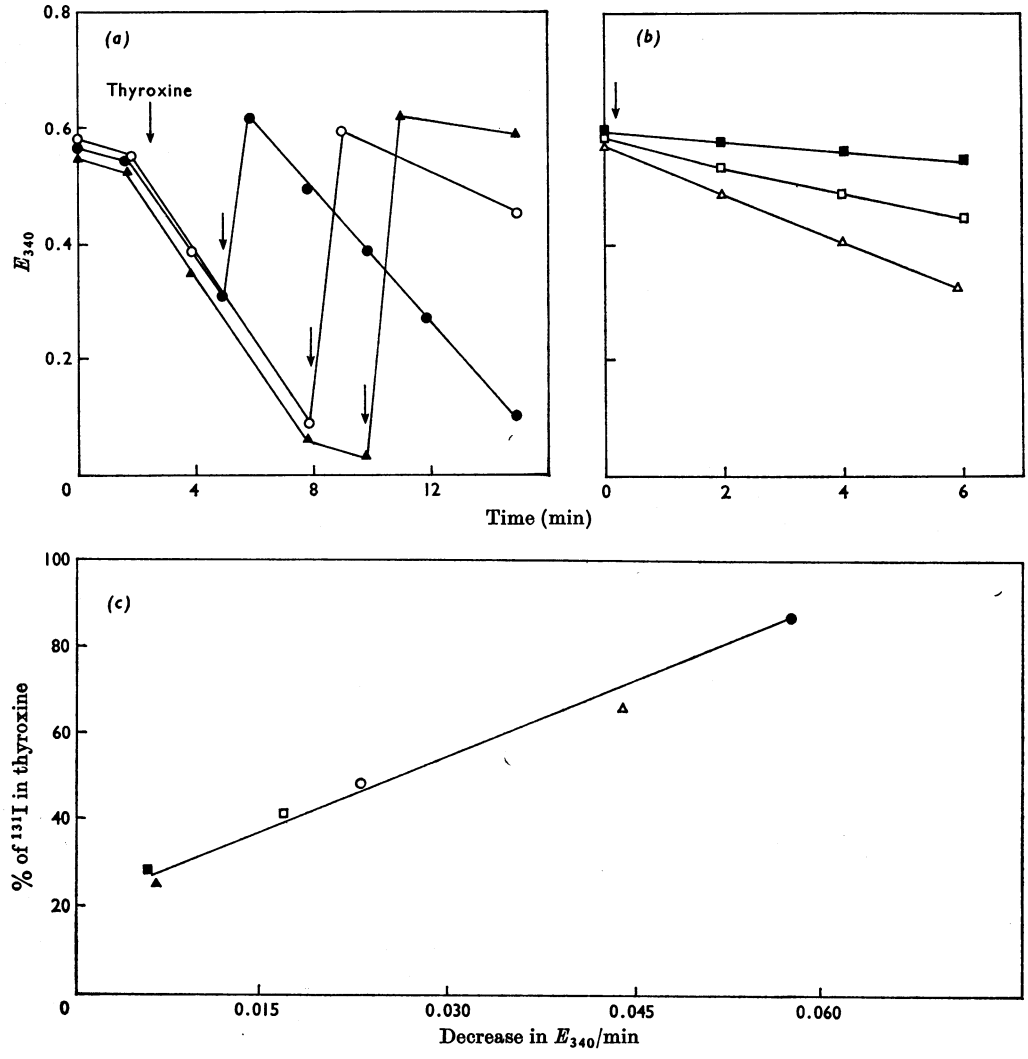

Fig. 6. (*a*) Relationship between the degree of oxidation of NADH by peroxidase+H_2O_2 as activated by thyroxine (first arrow), and the rate of oxidation of a second NADH sample added at the reaction time indicated by the subsequent arrows. The degree of deiodination of thyroxine was assessed by paper chromatography immediately before addition of the second NADH sample. (*b*) Rate of oxidation of an NADH sample, added (arrow) 15 (△), 30 (□) and 45 (■) s after preincubation of the peroxidase+H_2O_2 system with the thyroxine. A sample was withdrawn immediately before addition of NADH and the degree of deiodination of thyroxine was assessed as for (*a*). (*c*) Relationship between the degree of deiodination of thyroxine and the rate of oxidation of the second (Fig. 6*a*) or first (Fig. 6*b*) portion of NADH.

of thyroxine activated the oxidation of many successive NADH aliquots. (Results with 0.32 μmol of ICN were essentially the same, though the rate of NADH oxidation, both in the presence and absence of the peroxidase+H_2O_2 system, was much slower. For this reason, the experiment reported in Table 1 was done with much higher ICN concentrations.)

DISCUSSION

We have confirmed the following of Klebanoff's (1959*a*,*b*, 1960) observations. Thyroxine activates the oxidation of NADH [and D(−)-adrenaline] by peroxidase+H_2O_2, but does not do so in the absence of the oxidizing system. Both when the oxidizing capacity of the system increases and when

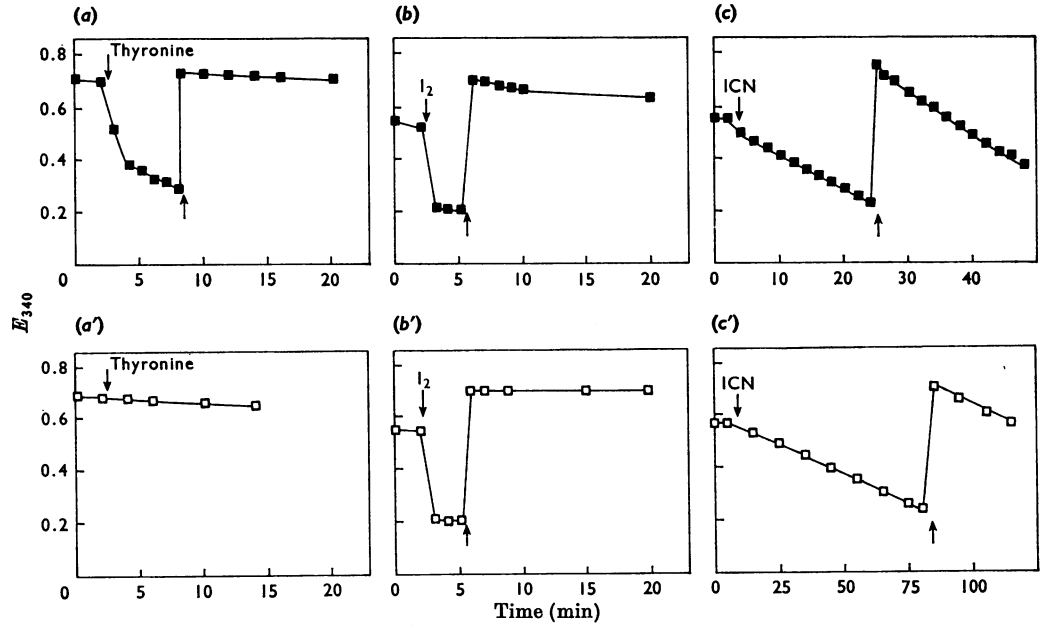

Fig. 7. Oxidation of NADH in the presence (*a*), (*b*) and (*c*) and absence (*a'*), (*b'*) and (*c'*) of the peroxidase+H_2O_2 system, after addition of thyronine (0.4 μmol), I_2 (0.30 μmol), or ICN (0.5 μmol). A second NADH sample (0.32 μmol) was added to the reaction mixture at the moment indicated by the arrow (↑).

it decreases because of the presence of potassium cyanide or catalase, the activating effect of a given amount of thyroxine changes in parallel. The addition of human plasma decreases the effect of thyroxine on the system. Incubation of thyroxine with peroxidase+H_2O_2 before the addition of NADH results in the loss of the activating effects of the hormone, and the ensuing NADH oxidation rate falls to values observed in the absence of the hormone.

We have, moreover, obtained direct experimental evidence in support of Klebanoff's (1959*b*) conclusion that the activating effect of thyroxine is catalytic. We have also shown that this catalytic effect, which requires the presence of the peroxidase+H_2O_2 system, may be mimicked with ICN, which, however, does not require this system. Though we have confirmed Klebanoff's (1959*a*) observation that thyronine affects the oxidation of NADH in the presence of peroxidase+H_2O_2, results presented above indicate that its action is not comparable with that of the iodine-containing analogue, thyroxine, in an important respect, namely the ability to catalyse the oxidation of many portions of NADH added successively. The effect of I_2 is also different from that of thyroxine and ICN, both in the absence or presence of the peroxidase+H_2O_2.

The above results therefore agree in general with Klebanoff's (1959*b*) conclusion that the activation by thyroxine of NADH oxidation by peroxidase+H_2O_2 'involves the cyclic oxidation and reduction of thyroxine, or a degradation product of thyroxine'. They also appear compatible with the idea that the moiety that is actually active in the system might be a degradation product of thyroxine, but not thyroxine itself, formed during the reaction. The important difference between thyroxine and thyronine revealed by the present study, and the similarities between thyroxine+peroxidase+H_2O_2 and ICN alone, would point to a very important role of the iodine atoms of the hormone. The system composed of thyroxine+peroxidase+H_2O_2 seems to act merely as a source of an oxidized form of iodine similar to that formed from ICN, and which would be the actual active moiety, stabilized by the presence of the hydrogen donor.

The above conclusions, however, are not clearly supported by the results of our direct measurements of the deiodination of thyroxine occurring during the reaction. Although it was known (Galton & Ingbar, 1963; Björkstén, Gräsbeck, Karlsson & Lamberg, 1963) that peroxidase+H_2O_2 deiodinate thyroxine, the possible protective effect of the NADH had not been assessed and the actual degree of deiodination of the hormone during the

Table 1. *Rate of oxidation of several succesive portions of NADH*

Successive portions of NADH (about 0.3 μmol each) were added in the presence of (*a*) 2 μmol of ICN; (*b*) 2 μmol of ICN and the peroxidase+H_2O_2 system; (*c*) 0.05 μmol of thyroxine and the peroxidase+H_2O_2 system. Experimental details are given in the text.

(*a*)			(*b*)			(*c*)		
Additions	Time (min)	E_{340}	Additions	Time (min)	E_{340}	Additions	Time (min)	E_{340}
1st NADH sample	0	0.61	1st NADH sample	0	0.62	1st NADH sample	0	0.70
—	1	0.60	Peroxidase+H_2O_2	1	0.61	Peroxidase+H_2O_2	1	0.69
ICN	2	0.60	ICN	2	0.60	Thyroxine	1.5	—
—	4	0.56	—	4	0.51	—	2	0.57
—	8	0.47	—	6	0.43	—	3	0.40
—	12	0.38	—	8	0.35	—	4	0.23
—	16	0.31	—	10	0.28			
—	20	0.25	—	12	0.23			
—	24	0.18	—	14	0.18			
2nd NADH sample	26	0.55	2nd NADH sample	15	0.54	2nd NADH sample	5	0.65
—	30	0.45	—	16	0.49	—	6	0.42
—	35	0.31	—	18	0.38	—	7	0.24
—	40	0.21	—	22	0.21			
16th NADH sample	230	0.81	23rd NADH sample	185	0.70	10th NADH sample	44	0.70
—	233	0.64	—	186	0.47	—	45	0.58
—	237	0.47	—	188	0.31	—	46	0.46
—	242	0.35	—	190	0.25	—	47	0.34
—	247	0.26	—	192	0.17	—	48	0.23

reaction was not known. On the one hand we have not found a linear relationship between the effect exerted by thyroxine on NADH oxidation by the peroxidase+H_2O_2 system and the degree of deiodination of [^{131}I]thyroxine. As we have indicated, the degree of deiodination of [^{131}I]thyroxine rarely exceeded about 20–25% until most of the NADH had been oxidized. Once this occurred, deiodination increased sharply and the ability of the system to activate the oxidation of newly added NADH was lost. These findings would not favour the conclusions discussed in the preceding paragraph. However, from experiments reported by Björkstén *et al.* (1963) and Björkstén (1966), with a comparable reaction system, it appears likely that a breakdown of the diphenyl ether structure has also occurred when the concentration of the hydrogen donor has decreased to the point where we find extensive deiodination of [^{131}I]thyroxine. Therefore, if this extensive deiodination of [^{131}I]-thyroxine were accompanied by breakdown of the thyroxine molecule, whereas the slight deiodination observed at E_{340} values above 0.25–0.20 were not, the latter would appear to be related to the intensity of the activating effect of thyroxine on NADH oxidation in a manner compatible with the conclusions of the previous paragraph.

But even if this were not so, would it be possible to reconcile two apparently contradictory conclusions? It has been suggested (Niemann, 1950; Jorgensen, 1964) that thyroxine affects redox reactions by acting as an electron carrier, passing through the semiquinonoid and quinonoid structures. However, the possibility should not be overlooked that the iodine atoms are also playing an important role, passing alternately through different stages of oxidation and reduction. These might be accompanied by looser and tighter binding respectively to the diphenyl ether structure, or by cyclic deiodination and iodination of the latter, until the concentration of the protective hydrogen donor (NADH in the present case) becomes so low that oxidative breakdown of the diphenyl ether structure occurs or the liberated iodine is oxidized to a higher state, no longer suitable for incorporation into the diphenyl ether structure. It has been suggested (Galton & Ingbar, 1961) that according to the principle of 'microscopic reversibility' (Ingold, 1953) the iodine liberated from the thyroxine structure during oxidation by peroxidase+H_2O_2 might be in the same form required for the iodination of phenolic compounds and, possibly, be similar to the one liberated from ICN (I^+ ?). Similar suggestions have been made by Michel (1964).

An alternative explanation is that the thyroxine is oxidized by peroxidase and H_2O_2 into a form that can in turn oxidize NADH. This form can also

undergo deiodination in a reaction that competes for it increasingly successfully as the concentration of NADH falls. Such an explanation is made more likely by the known susceptibility of quinones to nucleophilic displacements of such substituents as halogens. However, such an alternative would not take into account the similarities between the catalytic effect of ICN and that of the thyroxine + peroxidase + H_2O_2 system.

Even if present in the system, a 'reversible' deiodination–iodination of thyroxine might not have been observed by us because of the measures taken to avoid possible non-specific deiodination artifacts during the analytical procedures: these might have stabilized the iodinated form, and the ^{131}I would have been found as thyroxine unless the diphenyl ether structure had broken down. Obviously, this is an elusive problem to investigate.

The present investigation was done with the support of a grant from the J. March Foundation (Madrid) to T. J. The participation of Miss F. Martínez Capel in some experiments is gratefully acknowledged.

REFERENCES

Björkstén, F. (1966). *Biochim. biophys. Acta*, **127**, 265.
Björkstén, F., Gräsbeck, R., Karlsson, R. & Lamberg, B. A. (1963). *Annls Med. exp. Biol. Fenn.* **41**,156.
Braverman, L. E. & Ingbar, S. H. (1962). *Endocrinology*, **71**, 701.
Escobar del Rey, F. & Morreale de Escobar, G. (1964). *Proc. 2nd int. Cong. Endocrinology*, vol. 2, p. 1151. Amsterdam: Excerpta Medica Foundation.
Galton, V. A. & Ingbar, S. H. (1961). *Endocrinology*, **69**, 30.
Galton, V. A. & Ingbar, S. H. (1962). *Endocrinology*, **70**, 622.
Galton, V. A. & Ingbar, S. H. (1963). *Endocrinology*, **73**, 596.
Ingold, C. K. (1953). *Structure and Mechanism in Organic Chemistry*, p. 216. Ithaca: Cornell University Press.
Jolin, T., Morreale de Escobar, G. & Escobar del Rey, F. (1966). *Endocrinology*, **78**, 7.
Jorgensen, E. C. (1964). *Proc. Staff Meet. Mayo Clin.* **39**, 560.
Klebanoff, S. J. (1959*a*). *J. biol. Chem.* **234**, 2437.
Klebanoff, S. J. (1959*b*). *J. biol. Chem.* **234**, 2480.
Klebanoff, S. J. (1960). *Biochim. biophys. Acta*, **44**, 501.
Klebanoff, S. J. (1961). *Biochim. biophys. Acta*, **48**, 93.
Klebanoff, S. J. (1962*a*). *Biochim. biophys. Acta*, **56**, 460.
Klebanoff, S. J. (1962*b*). *Biochim. biophys. Acta*, **58**, 563.
Michel, R. (1964). In *Colloque sur les Hormones Thyroïdiennes*, p. 59. Ed. by Boulanger, P., Jayle, M. F. & Roche, J. Paris: Masson et Cie.
Morreale de Escobar, G. & Escobar del Rey, F. (1967). *Recent Prog. Horm. Res.* **23**, 87.
Morreale de Escobar, G., Llorente, P., Jolin, T. & Escobar del Rey, F. (1963). *Biochem. J.* **88**, 526.
Niemann, C. G. (1950). *Fortschr. Chem. org. NatStoffe*, **7**, 167.
Rall, J. E., Roche, J., Michel, R., Michel, O. & Varrone, S. (1963). *J. biol. Chem.* **238**, 1848.
Saunders, B. C., Holmes-Siedle, A. G. & Stark, B. P. (1964). *Peroxidase*, p. 136. London: Butterworths.
Taurog, A. (1963*a*). *Endocrinology*, **73**, 45.
Taurog, A. (1963*b*). *Endocrinology*, **73**, 57.